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Patentanmeldung Nr. Patent application No. Demande de brevet n°

98122412.4

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN  
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17/09/99



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**Sheet 2 of the certificate**  
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Phytosterol and/or phytostanol derivatives

The present invention relates to polyunsaturated fatty acid esters of phytosterols and/or phytostanols and their uses.

- 5        Phytosterols are plant sterols found, for example, in small amounts in vegetable oils such as corn, bean or other plant oils, where they occur as the free sterols, fatty acid esters, and glycosides. Phytosterols are structurally similar to cholesterol, the main differences occurring in carbon skeleton of their side chains. A number of different phytosterol structures are found in nature.
- 10      The most common ones are campesterol, beta-sitosterol and stigmasterol. Reduction of phytosterols yields saturated phytosterols, called phytostanols, such as campestanol or sitostanol, which also occur naturally in small amounts. A normal human diet typically leads to ingestion of less than one half gram a day of such substances in various forms.
- 15        It is known that ingestion of phytosterols and/or phytostanols in defined amounts, for example of several grams a day or more, can reduce blood serum cholesterol levels. It is assumed that free phytosterols and phytostanols inhibit the uptake of dietary and biliary cholesterol through displacement of cholesterol. However, generally only modest reductions of serum cholesterol
- 20      levels have been observed by adding free phytosterols or phytostanols to the diet.

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Arteriosclerosis is a leading cause of death in many parts of the Western world. It has been shown that low density lipoprotein (LDL) cholesterol is directly associated with the development of cardiovascular disease, whereas high density lipoprotein (HDL) cholesterol has an inverse relationship with cardiovascular disease development. People with combined hyperlipidemia run even higher risks of heart disease. Elevated blood serum levels of cholesterol and elevated levels of triglycerides are generally accepted both as causes and as indicators of the progression of cardiovascular disease. Thus lowering cholesterol and lowering triglycerides are both seen as desirable goals and major strategies for intervention. Many methods have been proposed to lower serum cholesterol, among them use of certain pharmaceutical agents and the ingestion of phytosterols in various forms. Likewise, many methods have been proposed to lower serum triglycerides, among them ingestion of polyunsaturated fatty acids (PUFAs) in various forms.

Physical properties are especially important in food applications. Properties of ingredients allow and limit the forms into which the products can be delivered e.g. in oils or butters. Further, properties such as solubility and melting point can affect acceptability of a food product by changing texture, mouth feel or taste in complicated, unpredictable ways. One problem with the use of free phytosterols has been their crystalline nature and limited solubility in oils. Generally, large amounts have been used to achieve effect on cholesterol levels but with resultant physical problems. Thus other forms have been sought.

WO 96/38047 discloses a fat-based food product comprising natural fat components which have a blood cholesterol lowering effect and wherein at least one component of tocotrienol, oryzanol and phytosterol is present physically mixed preferably with at least one component of PUFA-triglycerides. The phytosterols present are mainly in free phytosterol form in low, defined concentrations, and relatively insoluble. The resultant products are semi

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solids. Much higher amounts proportionally of the PUFA triglycerides to phytosterols are used. Effects of the mixtures on triglyceride levels are not described.

US Patent 4,588,717 discloses fatty acid esters of phytosterol as vitamin  
5 supplements or as diet pills, said esters being made from a phytosterol and a  $C_{18}$ - $C_{20}$  fatty acid. Included as such fatty acids are also the unsaturated acids linolenic, linoleic and arachidonic acid. It is generally known that these acids have almost no effect on the levels of triglycerides.

WO 97/42830 discloses the manufacture and the use of gels consisting of  
10 partly crystallised mixtures of natural food oils with low concentrations of sterols and sterol esters ( especially sitosterol and oryzanol ), and optionally monoglycerides, in defined ratios as a means to give firmness to edible liquid fats. Because of the low sterol and sterol ester content, such products of necessity require substantial volumes and additional caloric content to deliver  
15 phytosterols and phytosterol esters in amounts to effectively lower cholesterol.

WO 98/06405 discloses a method of reducing cholesterol in the blood-  
stream by administering beta-sitostanol with campestanol in defined ratios as fatty acid esters derived from vegetable oils.

20 US Patent 5,502,045 describes reduction of cholesterol absorbtion into the bloodstream by administering beta-sitostanol esters of  $C_2$ - $C_{22}$  acids derived from vegetable oils.

25 In Journal of Lipid Research, 1993, 34, 1535-1544, there are described and referenced experiments with human subjects fed mixtures of sitostanol esters made from rapeseed oil fatty acids. The phytostanol esters were found to reduce serum LDL cholesterol more effectively than free phytosterols despite being hydrolyzed during intestinal passage.

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In the European Journal of Clinical Nutrition, 1998, 52, 334-343 are presented results of human trials with margarines enriched with phytosterols and phytosterol esters. Plasma total and LDL cholesterol concentrations were shown to be reduced by sterol esters mixed with the margarines compared to  
5 controls with similar fatty acid profiles. All materials contained unsaturated fatty acid esters, especially those from oleic, linoleic or linolenic acid. No effect was seen on plasma triglyceride concentration with the sterol enriched margarines.

It has now been found that phytosterol and/or phytostanol esters made  
10 from phytosterols and/or phytostanols with certain polyunsaturated fatty acids are surprisingly effective in reducing both serum cholesterol and triglycerides. Such polyunsaturated fatty acids (n-3 fatty acids) are, for example, eicosapentaenoic acid (EPA) having five unsaturated carbon-carbon double-bonds or docosahexaenoic acid (DHA) with six unsaturated carbon-carbon double bonds.  
15 Said esters may be used as a combined cholesterol reduction agent and triglyceride lowering agent.

Physical properties of organic compounds such as physical state, melting point and solubility cannot be predicted reliably from chemical  
20 structures. These same properties contribute significantly to the acceptability of a food product by affecting texture, mouth feel or taste in complex and unpredictable ways. In the frame of the present invention there were synthesized esters of EPA and DHA with sitosterol, sitostanol and  
25 stigmasterol in pure form as well from mixtures of these sterols with other sterols and with mixtures of said acids with other fatty acids. Some of the compounds and mixtures were liquids whereas others were partly solid at room temperature or below. All were significantly more soluble in food oil than the corresponding phytosterols or phytostanols. For comparison there were  
30 also synthesized esters of sitostanol with mixed fatty acids containing significant levels of  $C_{18}$ - $C_{20}$  unsaturated fatty acids, especially linolenic acid, as obtained from rapeseed and it was found that the mixtures produced were largely crystalline at room temperature and below. Much more food oil was

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required to completely dissolve these esters compared to the esters prepared with EPA or DHA.

It was further found that the compounds according to the present invention offer unique physical advantages. The compounds offer a higher solubility in food oils than other phytosterol esters so far described, which is of advantage for the incorporation into a variety of food products. These materials allow co-delivery of phytosterols and/or phytostanols and selected PUFAs in their ester form in the highest concentration per unit volume possible. This is of advantage for incorporation into products where smaller volumes are important, such as in water dispersible formulations, or where additional non-essential food oils are undesirable. This presents physical advantages over simple mixtures or formulations of other phytosterols/phytostanols and/or their fatty esters with PUFAs and their normally available ester or triglyceride forms.

Accordingly, an object of the present invention are phytosterol and/or phytostanol esters made from phytosterols and/or phytostanols with PUFAs having from 18 to 22 carbon atoms and at least three unsaturated carbon-carbon double bonds. A further object of the present invention is the use of such phytosterol and/or phytostanol esters or mixtures thereof in human diet and diet-food for the purposes of lowering serum cholesterol levels and serum triglyceride levels in humans. The compounds according to the present invention are used preferably at total amounts of 1 to 10 grams per day of phytosterol ester and/or phytostanol ester content. A still further object of the present invention is the use of these phytosterol and/or phytostanol esters or mixtures thereof in formulations, in suitable physical forms, such as in capsules etc., as diet supplements or as ingredients in foods as well as these formulations per se.

Preferred phytosterols are beta-sitosterol or stigmasterol and campesterol or mixtures thereof. More preferred are beta-sitosterol and stigmasterol or mixtures thereof. Most preferred is beta-sitosterol. Preferred phytostanols

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are beta-sitostanol and campestanol or mixtures thereof. More preferred is beta-sitostanol. Preferred PUFAs are EPA and DHA

The compounds of the present invention need not be used in a pure state. Mixtures of the above substances where the sterol content is comprised  
5 predominantly of an ester according to the present invention and contains smaller amounts of phytosterols/phytosterols and phytosterol/phytosterol esters of other fatty acids are also within the scope of the present invention. Likewise mixtures of the above substances may also contain smaller amounts of free or PUFAs with lesser amounts of other saturated and unsaturated fatty  
10 acids.

The compounds according to the present invention can be prepared according to known methods. For example they can be obtained by esterifying a phytosterol/phytosterol with a n-3 PUFA in a known manner. Alternatively, they can preferably be prepared by interesterification of free phytosterols/  
15 phytosterols with esters of the n-3 PUFAs by heating in the presence of an interesterification catalyst, whereby (i) the interesterification is carried out solvent free, (ii) the fatty esters include suitable simple C<sub>1</sub>-C<sub>4</sub>-esters and triglycerides, (iii) the catalyst is a sodium alkoxide of a C<sub>1</sub>-C<sub>4</sub>-alcohol. The reaction is suitably conducted by heating the mixture at 80-140°C at a  
20 pressure of 133-6650 Pa whereby the reaction preferably is carried out with a stoichiometric amount to an excess of the PUFA ester. The following examples further illustrate the invention.

#### Example 1

25 To a mixture of 0.91 g of docosaheptaenoic acid (purity: 90%), 1.03 g of stigmaterol (purity: 95%) and dimethylaminopyridine (50 mg) in 18 ml of dry dichloromethane was added a solution of dicyclohexylcarbodiimide (0.63 g) in 5 ml dichloromethane. After 4 hours stirring at room temperature, the reaction was complete. Then, methanol (0.5 g) and acetic acid (0.25 g) were  
30 added and the mixture stirred for a further one hour. The mixture was cooled

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to 0°C, filtered, and the solids rinsed with hexane (3 x 25 ml). The solvent was removed under reduced pressure and the residue flash chromatographed on silica to yield a pure fraction of 1.0 g of stigmasterol docosahexaenoate as a colourless oil with consistent NMR and IR data. This substance remained an oil when stored for several weeks at room temperature and when cooled for several weeks at -20°C.

#### Example 2

Analogous to Example 1, stigmasterol eicosapentaenoate was prepared from eicosapentaenoic acid (purity: 90%) and stigmasterol. Stigmasterol eicosapentaenoate (1.46 g) was obtained as a colourless oil which remained liquid within a temperature range of 20°C and -20°C.

#### Example 3

Analogous to Example 1 a mixture of eicosapentaenoic acid-docosahexaenoic acid esters of stigmasterol was prepared from stigmasterol with a mixture of 49% eicosapentaenoic acid and 27% docosahexaenoic acid. The mixture of the esters of stigmasterol was obtained as a colourless oil which remained liquid within a temperature range of 20°C and -20°C.

#### Example 4

Analogous to Example 1 stigmastanol docosahexaenoate was prepared from stigmastanol (purity: 95%) and docosahexaenoic acid (purity: 90%). Stigmastanol docosahexaenoate was obtained as a slightly coloured oil which remained liquid between 20°C and -20°C.

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#### Example 5

Analogous to Example 1 stigmastanol eicosapentenoate was prepared from stigmastanol and eicosapentaenoic acid. Stigmastanol eicosapentenoate was obtained as a slightly yellowish oil which remained liquid within the temperature range of 20°C and -20°C.

#### Example 6

Analogous to Example 1 a mixture of stigmastanol eicosapentaenoic acid and docosahexaenoic acid esters was prepared from stigmastanol and a mixture of 49% eicosapentaenoic acid with 27% docosahexaenoic acid. A mixture of stigmastanol eicosapentaenoic acid and docosahexaenoic acid esters was obtained as a colourless oil which became turbid when stored at 20°C and partly solid at -20°C.

#### Example 7

Analogous to Example 1 a mixture of sterol PUFA esters was prepared from a mixture of beta-sitosterol, campesterol, and stigmasterol and a mixture of 49% eicosapentaenoic acid with 27% docosahexaenoic acid. A mixture of sterol PUFA-esters was obtained as a turbid oil containing some solids at 20°C and partly solid at -20°C.

#### Example 8

Analogous to Example 1, a mixture of stigmastanol unsaturated fatty esters was prepared from stigmastanol and a mixture of fatty acids obtained from basic hydrolysis of a commercial food sample of Swiss rapeseed oil (9% saturated, 61% monounsaturated, 30% polyunsaturated triglycerides). A mixture of stigmastanol unsaturated fatty esters was obtained as a colourless oil which slowly crystallised at room temperature. At -20 C the material was essentially solid.

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Example 9

Solubilities of materials made according to the procedures described in Examples 1-8, as well as the parent sterols were assessed in a commercial sample of Swiss rapeseed oil by alternately adding small increments of oil at room temperature to weighed amounts of sterol esters and agitating for 5 minute periods until solution was attained. Minimum starting ratio was about 1:1 and trials were discontinued at above 10:1.

10	<u>material</u>	<u>solubility g oil /g material</u>
	stigmasterol docosahexaenoate	miscible > 1
	stigmasterol eicosapentenoate	miscible > 1
	stigmasterol EPA-DHA ester mixture	miscible > 1
	stigmastanol docosahexaenoate	miscible > 1
15	stigmastanol eicosapentaneate	miscible > 1
	stigmastanol EPA-DHA ester mixture	soluble > 4
	sitosterol sterols mix EPA-DHA ester mixture	miscible > 1
	stigmastanol rape-seed ester mixture	insoluble > 10
	stigmasterol	insoluble > 10
20	stigmastanol	insoluble > 10
	docosahexaenoic acid ethyl ester 90%	miscible > 1
	EPA ethyl ester 90%	miscible > 1

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1. Phytosterol and/or phytostanol esters made from phytosterols and/or phytostanols with polyunsaturated fatty acids, characterised in that the poly-  
5 unsaturated fatty acid has from 18 to 22 carbon atoms and at least three unsaturated carbon-carbon double bonds.

2. Esters according to claim 1, wherein the phytosterol is beta-sitosterol, stigmasterol or campesterol or a mixture thereof, preferably beta-sitosterol or  
10 stigmasterol or a mixture thereof, most preferably beta-sitosterol.

3. Esters according to claim 1 or claim 2, wherein the phytostanol is campestanol or beta-sitostanol or a mixture thereof, preferably beta-sitostanol.

15 4. Esters according to any one of the claims 1 to 3, wherein the polyunsaturated fatty acid is eicosapentaenoic acid or docosahexaenoic acid.

5. The use of esters according to any one of claims 1 to 4 in human diet and diet-food for the purposes of lowering serum cholesterol levels and serum  
20 triglyceride levels in humans.

6. The use of esters according to any one of claims 1 to 4 in formulations, in suitable physical forms, preferably in capsules, as diet supplements or as ingredients in foods.

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7. Formulations, in suitable physical forms, preferably in capsules, diet supplements or foods containing esters as claimed in any one of claims 1 to 4.

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8. A process for preparing esters according to any one of claims 1 to 4, comprising esterifying a free phytostero/phytostanol or a mixture thereof with a n-3 polyunsaturated fatty acid having from 18 to 22 carbon atoms and at  
5 least three unsaturated carbon-carbon double bonds or a mixture thereof in a known manner.

9. Esters according to any one of the claims 1 to 4, characterized in that they have been obtained by interesterification of free phytosterols/phyto-  
10 stanols with fatty esters of the n-3 polyunsaturated fatty acids by heating in the presence of an interesterification catalyst, whereby (i) the interesterification is carried out solvent free, (ii) the fatty esters include suitable simple  $C_1$ - $C_4$ -esters and triglycerides, (iii) the catalyst is a sodium alkoxide of a  $C_1$ - $C_4$ -alcohol, whereby the reaction is suitably conducted by heating the mixture at  
15 80-140°C at a pressure of 133-6650 Pa and whereby the reaction is carried out with a stoichiometric amount to an excess of the PUFA ester.

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Abstract

Phytosterol and/or phytostanol esters with polyunsaturated fatty  
5 acids having from 18 to 22 carbon atoms and at least three carbon-carbon  
double bonds are effective in reducing both serum cholesterol and triglycerides

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